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14. ABSTRACT The primary aim of this project is to laser microdissect stage I lung cancer samples, perform microRNA profiling of the epithelial and stromal components and develop component specific signatures of prognosis. This report summarizes the work performed on the project so far. Data analyses indicate that there is a significant difference in the microRNA expression profile of epithelia and stroma in NSCLC tumors and that these profiles have prognostic import with stroma having more prognostic value than epithelia. Also, the fold changes seen along with variability of in-situ hybridization (ISH) measurements do not support pursuing the development of prognostic ISH biomarkers. Based on the data, two microRNAs were chosen for further study. miR-372 overexpression makes cell lines more aggressive with increased migration and invasion. miR-146b was chosen as the stromal miRNA for further study. A no-cost extension was granted to complete these experiments.					
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Introduction:

Even stage I lung cancer patients have an unacceptably high rate of recurrence (~35%)¹. A prognostic assay can help identify patients for intensified treatment such as adjuvant chemotherapy. Our previous data has demonstrated the potential of microRNA profiling of whole tumors to prognosticate early non-small cell lung cancer (NSCLC)². However, some of the prognostic “signal” may be masked by varying composition of whole tumors with respect to their epithelial and stromal components. In this project, we intended to perform laser capture microdissection of lung cancer specimens to separate out the epithelial and stromal components of tumors and perform microRNA profiling of these separate components to try to improve our prognostic assay and to identify specific cancer epithelial microRNA of biological import. In last year’s progress report the following conclusions were reached:

- 1) The epithelial and stromal components of tumors are significantly different in terms of microRNA expression.
- 2) Most prognostic information arises from the stroma.
- 3) Fold change differences obtained from component-specific microRNA profiling are in the same range as that of whole tumor microRNA profiling.
- 4) ISH is not a good technique for the development of robust prognostic biomarkers for NSCLC.

Based on the statement of work, tasks 1 and 2 were completed. This progress report describes progress made in task 3: **To study the role of two miRNAs predicting prognosis in regulation of the phenotype of lung cancer.**

3a. Further higher-level analyses of the microarray data will be performed to select two microRNAs suitable for further functional study.

As the microarray data suggested that a significant amount of prognostic signature came from the stroma, the decision was made to delineate the biological significance of one epithelial microRNA and one stromal microRNA. Selection of the epithelial microRNA was performed in the following fashion:

1. The microRNAs with the greatest difference between patients with and without recurrence in the epithelial components were identified.
2. This list was refined further based on other microRNA profiling studies to select the top prognostic microRNAs and ranked accordingly.
3. A literature search was performed to identify those microRNAs whose biological role has already been elucidated and these were excluded from further study.

Based on the above steps miR-372 and miR-486 were selected.

3b. Creation of stably transfected cell populations: The selected miRNAs will be cloned into transfer vectors, lentivirii generated and used to develop stably transfected populations: Stably transfected population of A549 cells overexpressing these miRs were created and their study initiated. During this time, publications elucidating the role of miR-486 were published; therefore, the study of this miR was stopped. The in-vitro analyses of miR-372 was continued.

3c. Cell line experiments to evaluate phenotype (28-33 months):

- a) Cell proliferation and colony formation: There is no difference in proliferation of A549 cells overexpressing miR-372 (AE3) vs. control (AE0). (Figure 1). Similarly, there was no difference in the ability of miR-372 to influence colony formation by A549.

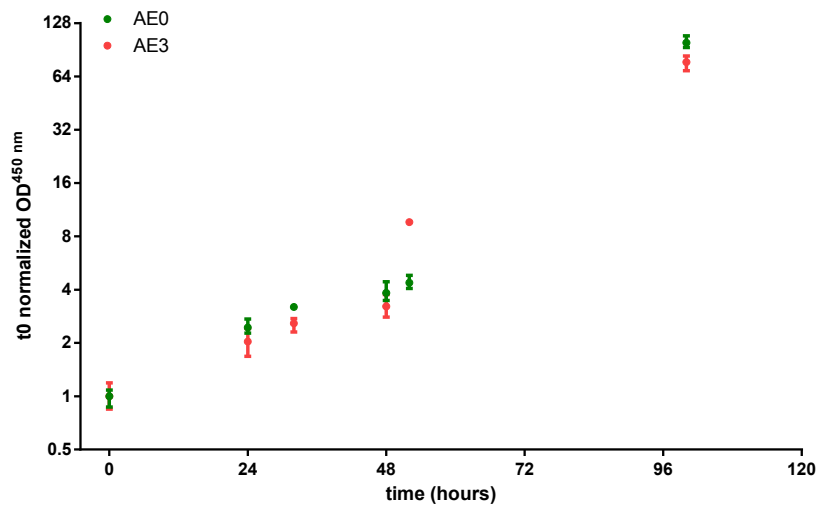


Figure 1. Comparison of cell proliferation of A549 cells overexpressing miR-372 (AE3) vs. control (AE0).

- b) Cell invasion assay: A549 cells overexpressing miR-372 have a significantly higher cell invasion than controls (Figure 2).

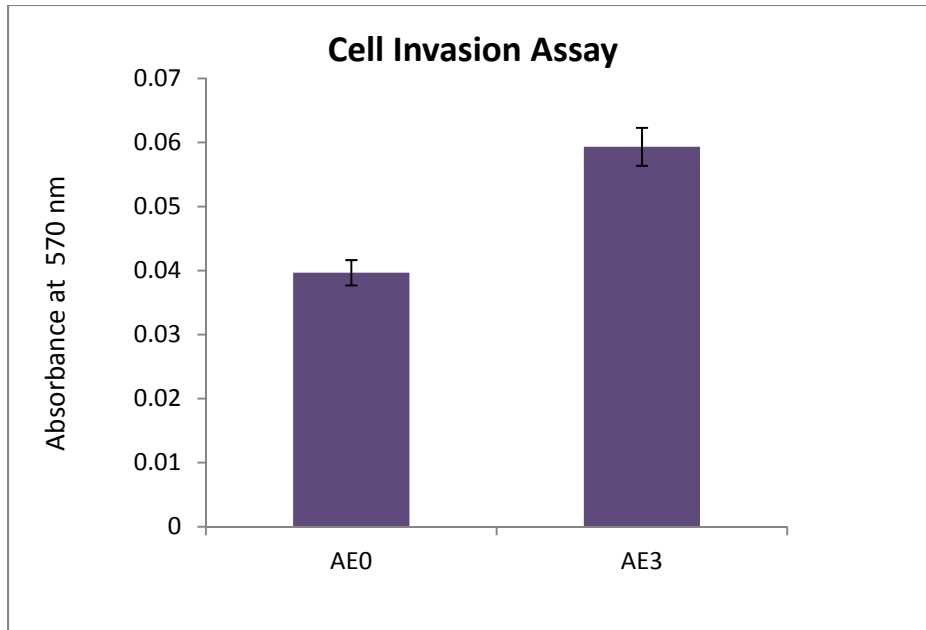


Figure 2. Comparison of cell invasion of A549 cells overexpressing miR-372 (AE3) vs. control (AEO).

- c) Cell migration by scratch assay: A549 cells overexpressing miR-372 exhibit more cell migration as evidenced by a scratch assay (Figure 3).

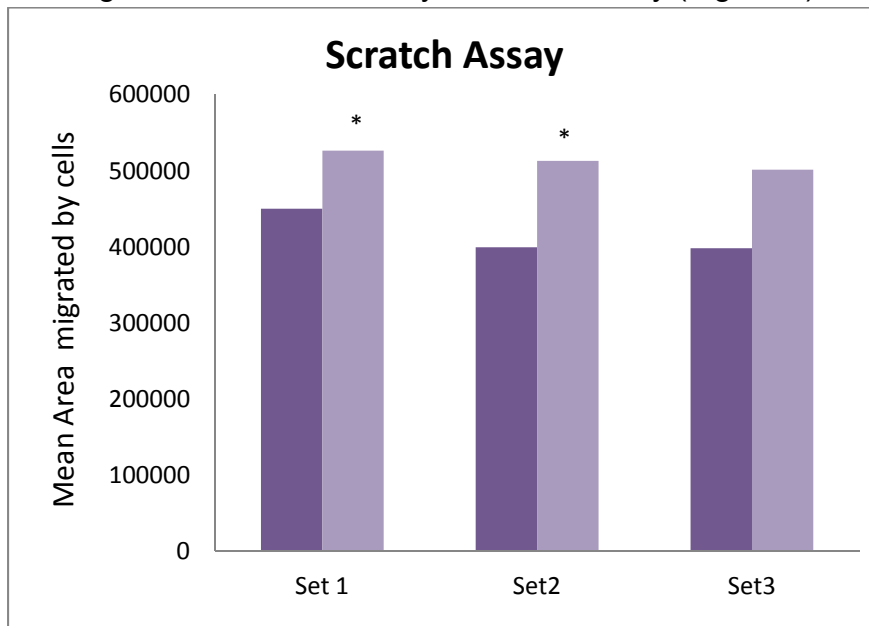


Figure 3. Comparison of cell migration of A549 cells overexpressing miR-372 (AE3) vs. control (AEO).

- d) Cell cycle analysis: A549 cells overexpressing miR-372 have a greater proportion of cells in the S phase than controls (Figure 4).

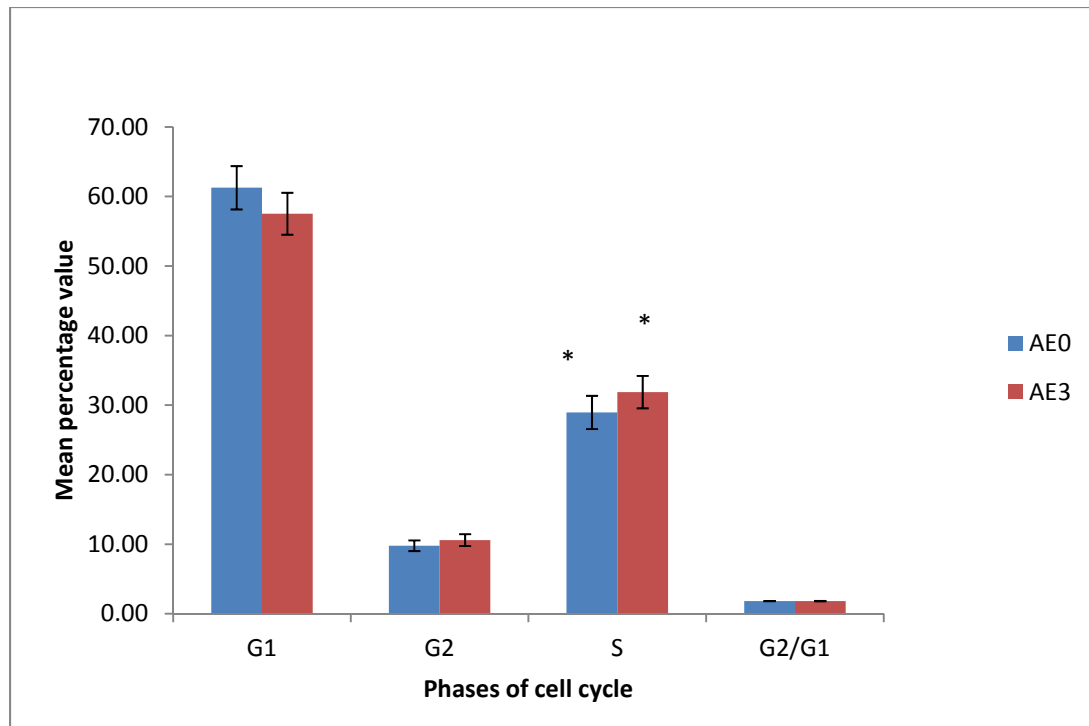


Figure 4. Cell cycle analysis of A549 cells overexpressing miR-372 (AE3) vs. control (AE0).

Selection of a stromal microRNA for further study:

Based on our component profiling experiments, several potential candidates were available for study. Of these, we chose miR-146b for further study based on the following reasons:

- 1) miR-146b has been shown, by us as well as others, to be very strong prognostic marker.^{2,3}
- 2) We have demonstrated convincingly that miR-146b does *not* alter epithelial lung cancer cells⁴.
- 3) miR-146b is very strongly expressed in macrophage-monocyte lineages compared to all other tissues⁵.

Based on the data above, we hypothesized that miR-146b influences the differentiation of M1 to M2 macrophages. To test this hypothesis, we plan to use a well published model that separates macrophages from peripheral blood⁶. These macrophages are then exposed to GM-CSF to differentiate them into CD-206+ve M2 macrophages. We have replicated this model in our laboratory. We now plan to introduce miR-146b into these macrophages and assess the ability of miR-146b to influence this phenotype.

Planned experiments:

3d. Xenograft formation assays (March 2014 – June 2014): Xenograft formation assays will be performed here in triplicate. An existing IACUC protocol will be amended to perform these experiments.

3e. Identification of miRNA targets (Dec 2013 – March 2014): 3'UTR reporter assays will be performed to identify bonafide miRNA targets to explain the altered phenotypes.

3f. Assessment of the role of miR-146b in altering macrophage differentiation: miR-146b constructs will be used to study the role of M1 to M2 macrophage conversion as described above (Oct 2013 – March 2014).

Key Research Accomplishments:

- Optimization of RNA yield from laser microdissectates of paraffin embedded non-small cell lung cancer specimens.

Reportable Outcomes:

- Component specific microRNA profiles of NSCLC have been obtained for the first time. These profiles show that much greater prognostic information comes from tumor stroma rather than tumor epithelium.
- MiR-372 overexpression makes lung cancer cells acquire a more “invasive” phenotype without significantly impacting cell proliferation, in keeping with microRNA profiling experiments.

Conclusion:

In the past year we have:

- 1) Demonstrated that miR-372 makes lung cancer cells more “invasive”
- 2) We have identified a stromal microRNA to study further and have established a model system to study it in.

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